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The Histochemical Demonstration of Protein-bound Sulfhydryl Groups and Disulfide Bonds in Human Hair by a New Staining Method (DACM Staining)

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A new fluorogenic maleimide DACM, i.e., N(7-*d*imethyl *a*mino-4-methyl *c*oumarinyl) maleimide, does not fluoresce by itself. It is specifically combined with —SH groups and becomes fluorescent (λ ex: 400 nm, λ em: 485 nm). At this range of excitation the frozen skin section has no native fluorescence because the emission maximum of DACM does not overlap with any of aromatic residues of proteins such as tryptophane. S—S groups can be demonstrated with DACM by inhibiting native —SH and then reducing SS to —SH. —SH was generally abundant at the bulb region. —SH of hair cortex was more concentrated at keratogenous zone. Further up in the follicle, —SH of hair cortex was gradually decreased, although at the region of isthmus, —SH reaction of the hair cortex was still moderately strong. Outer root sheath had —SH from the upper bulb to the surface of the epidermis. On the other hand, no S—S was demonstrable at the bulb region. At the level of keratogenous zone, however, S—S linkages of hair cortex and inner root sheath began to appear and further up in the follicle S—S linkages were increased gradually. Outer root sheath had no S—S linkages by DACM staining up until it is keratinized along the hair canal in the upper follicle. The concentration of —SH and S—S thus seemed to be reciprocal; —SH is found in non-keratinized tissues, whereas S—S is abundant in keratinized areas. These findings are at some variance with conventional data, which suggest, for example, that —SH groups disappear suddenly above the keratogenous zone.

The distribution of sulfhydryl groups and S—S covalent linkages is one of the important problems in the process of hair keratinization. Recently, Kanaoka and Sekine synthesized a new fluorescent thiol reagent DACM, i.e., N(7-*d*imethyl-*a*mino-4-methyl *c*oumarinyl) maleimide [1]. This reagent does not fluoresce by itself, but becomes fluorescent when it is specifically combined with sulfhydryl groups. Usefulness of this reagent has been already shown in a number of biological materials [2,3]. We applied a histochemical method based on DACM to localize and determine the concentration of —SH groups and S—S covalent linkages in normal epidermis [4,5] and squamous cell carcinoma [6]. S—S covalent linkages can be demonstrated with DACM by inhibiting native —SH groups and then reducing S—S to —SH. As an extention of these previous works, we studied the localization and concentration of —SH groups and S—S covalent linkages in human hair.

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Abbreviations:
DACM: N(7-dimethyl-amino-4-methylcoumarinyl) maleimide
—SH: Sulfhydryl
—SS: Disulfide
TAS: Tris acetate saline buffer

MATERIALS AND METHODS

Biopsy specimens were obtained from the scalp of 5 healthy males and 3 females ranging in age from 20 to 40 yr. More than 100 hairs were used. All tissues were immediately frozen at −20°C and sectioned at 2–4 μ in Slee HR Mark II cryostat. Sections were air-dried and used for histochemical staining of —SH groups and S—S covalent linkages. All the hairs used were in anagen stage of hair cycle and qualitative and quantitative variability was minimal.

Properties of DACM

The newly synthesized compound DACM* has very high sensitivity, a large molecular extinction coefficient and a high quantum yield. DACM was kept in cold acetone solution or in cold acidic buffer, because it becomes fluorescent in alkaline pH (8.0). We used pH 6.8 tris acetate saline containing 0.85% NaCl (TAS) for staining the sections; under this condition natural decay of the reagent did not occur. Other chemicals such as N-ethylmaleimide (NEM), ethylenediaminetetra acetate (EDTA), and dithiothreitol (DTT) were obtained from Sigma Chemical Company (St. Louis, Mo.).

Staining Method for Protein Bound Free —SH Groups

Tissue sections were rinsed in cold solution (4°C) of TAS for 5 min to remove free cysteine, cystine and glutathionine, and incubated with TAS containing 0.01 mM of DACM at room temperature for 1 min. After incubation, each section was washed by cold TAS and observed by microspectrofluorometer. For observation we used an excitation filter which excites 400 nm and barrier filter which eliminates wave lengths below 460 nm.

Staining Method for S—S Covalent Linkages

After blocking of —SH by 0.15 M NEM in TAS at 37°C for 3 min, each section was washed with cold TAS. Subsequently, S—S of these sections was opened (reduced) to —SH by 0.5 mM EDTA and 40 mM DTT in TAS at 37°C for 3 min. After opening S—S linkages, —SH groups were stained with DACM in the same manner as described above. Specificity of —SH inhibition by NEM was complete; this is understandable because DACM was synthesized as a maleimide analog of NEM.

In this study we stained transverse sections and longitudinal sections of human hair. Transverse sections were made from the bulb, through the keratogenous zone, isthmus and up to the infundibulum. —SH and S—S contents were compared in serial sections in both transverse and longitudinal sections and photographic recordings using Nikon fluorescence microscope were made at the same magnification and exposure time to most of the sections.

Three independent observers examined each section and recorded the result in 5 categories; namely very strongly positive (++++), strongly positive (+++), moderately positive (++), weakly positive (+), and negative (−).

RESULTS

Diagrams I, II and Tables I, II summarize the following descriptions.

In general, greenish fluorescence denoted a strong reaction and bluish fluorescence indicated a weak reaction.

Sulfhydryl (—SH) staining

Epidermis: In the human interfollicular epidermis an abundance of —SH groups was found in the cytoplasm of living

* Available from Teika Seiyaku Co., Ltd., 250 Arakawa, Toyama, Japan.

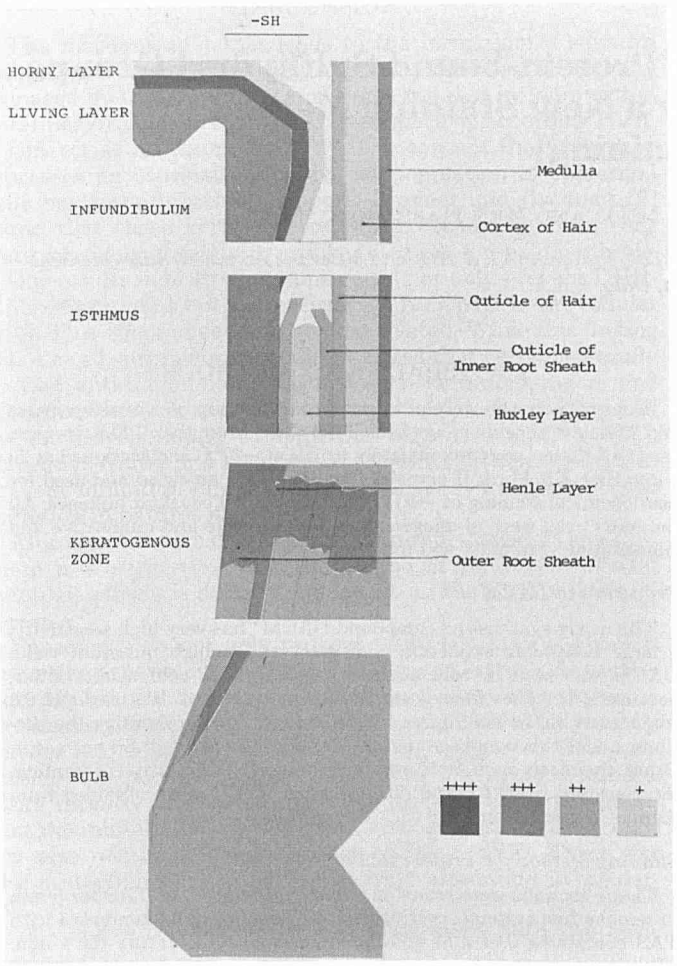


Diagram I. Schematic representation of typical —SH distribution in human hair and its follicle.

layers (basal, spinous and granular layers) (Fig 1*A*). The fluorescence was more concentrated along the cell periphery, i.e., either cell membrane or intercellular spaces. The intensity of fluorescence increased at the border zone of living and horny layers (Fig 1*A*). In horny layer, the fluorescence of the cell membrane or intercellular spaces was noticed in the lower layers but decreased gradually toward outer layers (Fig 1*A*).

Hair follicle

The outer root sheath was strongly reactive for SH groups from upper bulb to infundibulum. The intensity of the reaction was almost same as in the living layers of the epidermis; thus in longitudinal sections a continuous fluorescence from above the bulb to the surface epidermis was observed (Fig 1*B*, 1*C*, 1*D*). The cell periphery had a stronger fluorescence than general cytoplasm of those cells. This was particularly noticed where the fluorescence was weak (Fig 2*A*, 2*B*, 2*C*).

In the region of the bulb, inner root sheath, cuticle of hair, hair cortex and hair medulla had a moderate fluorescence, while the hair papilla had only a weak or negative fluorescence (Fig 1*D*). The distribution and intensity of —SH groups in these layers were more or less uniform in the bulb (Fig 1*D*). At the level of keratogenous zone, inner root sheath and cortex of hair became more reactive (Fig 1*D*, 2*C*). Henle's layer became fluorescent earlier and was more reactive than Huxley's layer (Fig 1*D*, 2*C*). Generally, cell periphery had stronger fluorescence than cytoplasm in these layers, as described in the epidermis and outer root sheath above (Fig 2*C*).

Further up in the follicle, Huxley's layer, cuticle of hair and cuticle of the inner root sheath became more reactive (Fig 2*B*). Toward the epidermis the fluorescence of all layers gradually decreased except the inner root sheath which contained Henle's layer. Detached cells of the inner root sheath showed irregular but often strong residual fluorescence (Fig 2*A*). The cuticle of the cortex had a strong fluorescence above the keratogenous zone (Fig 2*A*, 2*B*). Cortex of the hair had a moderate but irregular reaction in the upper follicle particularly in the center corresponding to medulla. However, the intensity of the reaction was less than in keratogenous zone (cf. Fig 2*A* with Fig 2*B*). Sebaceous glands were reactive only in cell wall but keratinized duct near the outlet to the follicle was strongly fluorescent (Fig 1*B*).

S—S Staining

Epidermis: In the sections previously blocked with NEM the epidermis was completely negative for —SH by DACM staining. After the reduction of S—S to —SH, positive fluorescence for —SH (thus for S—S) was noted in the stratum corneum. The localization of S—S linkages were not distinctive in living layers (basal to granular layers). Strong fluorescence of cell periphery (cytomembrane or intercellular spaces) appeared suddenly at the junction of living and horny layer (Fig 3*A*).

Hair follicle: Outer root sheath was nonreactive throughout its lower levels (Fig 3*A*, 3*B*, 3*C*). However, as the cells of outer root sheath began to keratinize, it became reactive along the hair canal at the level of isthmus.

In the hair bulb the cells of outer root sheath, inner root sheath, cortex of the hair and hair medulla had no fluorescence

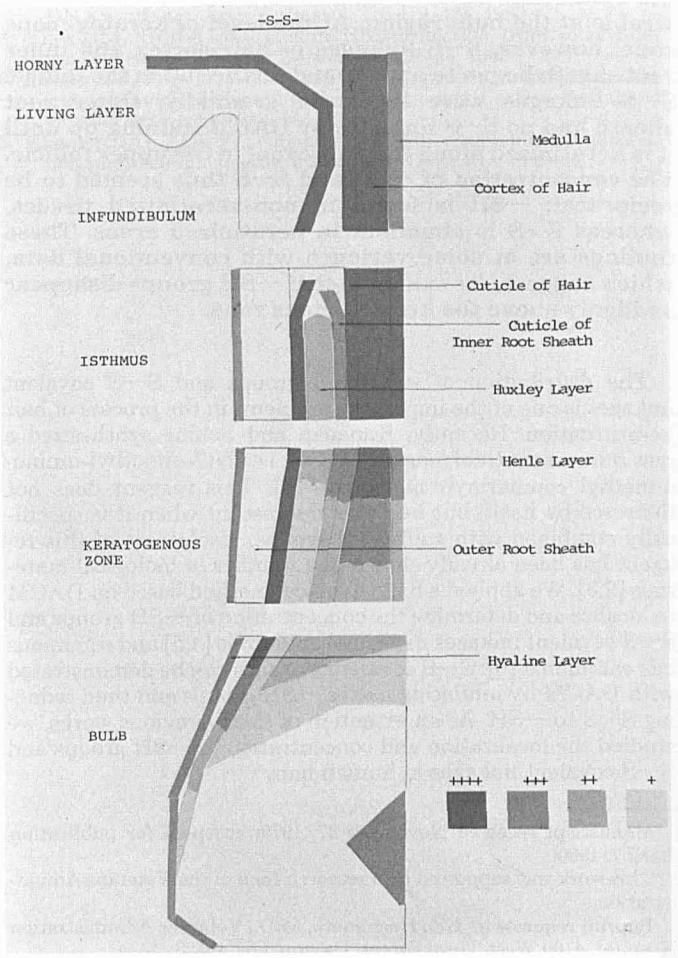


Diagram II. Schematic representation of typical S—S distribution in human hair and its follicle.

TABLE I. *-SH and S-S distribution in various layers of hair follicle*

	Hair papilla	Medulla	Cortex	Cuticle of cortex	Cuticle of inner root sheath	Huxley	Henle	Outer root sheath
				-SH				
Bulb	−	+	++	++	++	++	++	+
Keratogenous zone	/	++	++~+++	++~+++	++~+++	++~+++	+++	−
Isthmus	/	+++	+++	++	++	+	++	+++
Infundibulum	/	+++	+	++	/	/	/	+++
				S-S				
Bulb	+	−	−	−	−	−	−	−
Keratogenous zone	/	++~+++	++~++++	++~++++	+~+++	+	++++	−
Isthmus	/	+++	++++	++++	+++	+~++	++++	−
Infundibulum	/	+++	++++	++++	/	/	/	−

Note: (++++) Very strong fluorescence.
(+++) Strong fluorescence.
(++) Moderately strong fluorescence.
(+) Weak fluorescence.

TABLE II. *-SH and S-S distribution at various levels*

	-SH	S-S
Bulb		
Hair papilla	−	+
Medulla	+	−
Cortex	++	−
Cuticle of cortex	++	−
Cuticle of inner root sheath	++	−
Huxley	++	−
Henle	++	−
Outer root sheath	+	−
Keratogenous Zone		
Medulla	++	++~+++
Cortex	++~+++	++~++++
Cuticle of cortex	++~+++	++~+++
Cuticle of inner root sheath	++~+++	++~+++
Huxley	++~+++	+
Henle	+++	++++
Outer root sheath	+++	−
Isthmus		
Medulla	+++	++++
Cortex	+~++	++++
Cuticle of cortex	++	++++
Outer root sheath	+++	−
Cuticle of inner root sheath	++	+++
Huxley	+	+~++
Henle	++	++++
Infundibulum		
Medulla	+++	+++
Cortex	+	++++
Cuticle of cortex	++	++++
Outer root sheath	+++	−
		++ along *hair canal*
Epidermis		
Living layers (basal, prickle & granular)	+++~++++	−
Junction between living layer and horny layer	++++	−
Horny layer	±	++++

Note: Symbols are same as in Table I.

(Fig 3*C*). In this area only hyaline membrane and hair papilla were weakly to moderately fluorescent (Fig 3*C*). At the region of keratogenous zone Henle's layer became reactive for S—S and the cortex also fluoresced. The reaction in cortex was less intense than that of Henle's layer (Fig 3*C*, 4*B*). Above the keratogenous zone, in addition to Henle's layer and hair cortex, the cuticles and Huxley's layer became reactive (Fig 3*B*, 4*A*). Huxley's layer was weakly fluorescent (Fig 4*A*). At this level, the fluorescence of cortex became as strong as Henle's layer (Fig 4*A*). Further up in the follicle, the fluorescence of these layers became more intense. Among others, the strongest fluorescence was seen still in Henle's layer.

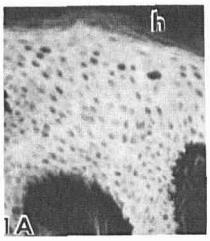
It was difficult to differentiate cuticles of inner root sheath and cuticle of the hair. Above the keratogenous zone we could only recognize one strong fluorescent band which seemed to represent both cuticles. At the isthmus, however, we could differentiate cuticle of inner root sheath and cuticle of hair. Both cuticles had a strong fluorescence (Fig 3*B*). At the level of isthmus strongest fluorescence was seen in the hair cortex and inner root sheath, especially still in the Henle's layer. The fluorescence of these layers was almost same as that of the horny layer of the epidermis (cf. Fig 3*B* with 3*A*).

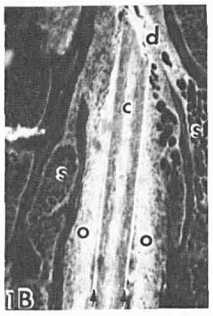
DISCUSSION

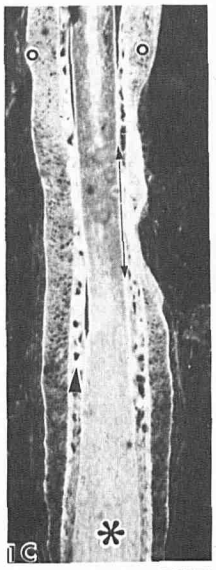
Barrnett-Seligman method [7] has been standard to detect —SH groups and S—S linkages by histochemical staining. This method uses 2,2′-dihydroxy-6,2′-dinaphthyl disulfide which reacts with active —SH groups and form a colorless substance. Unbound excess reagent and by-products should be removed by passage through alcohols and ether. Subsequently, the reaction product is coupled with azo dye and the excess dye is washed again. The difficulty of this method is extraction of by-product or avoidance of nonspecific binding; therefore nonspecific staining might occur. Our new staining method is very simple; i.e., one drop of DACM on a frozen section to be stained is all needed. Within 10 seconds, all stainable structures become fluorescent under the direct microscopic observation. The staining is very specific as shown previously [1,4]. The emission maximum is distinct from that of the aromatic residues of protein; thus native fluorescence of elastic fiber, for example, could be eliminated. It is soluble in water until combined with —SH. Photostability of DACM—SH is very high. These characteristics of DACM were uniquely suited as a histochemical agent for the study of —SH in hair; namely, adequate penetration into complicated structures of the hair, high specificity, bright fluorescence, specific *in situ* demonstration of —SH by insoluble reaction product, and recordability with relatively slow speed color film without fear of fading of fluorescence.

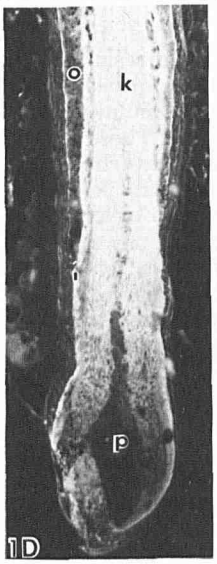
Regarding S—S linkages, they were only barely detectable in the living layer. These appeared suddenly at the junction of living and horny layers, and the fluorescence was localized to the membrane or intercellular spaces.

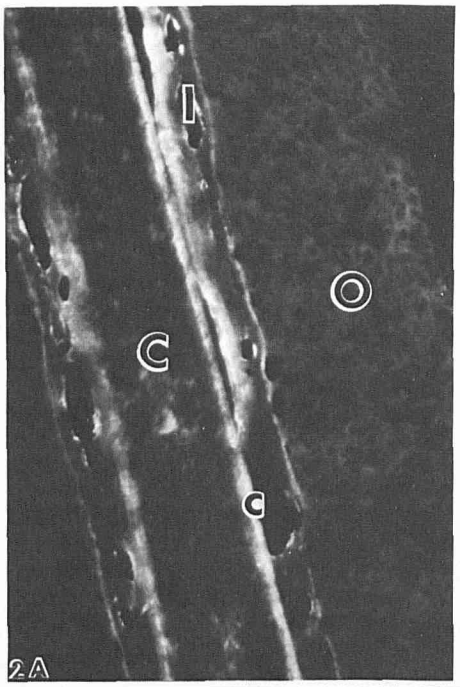
It is tempting to postulate that a concentration of —SH at the cell periphery is suddenly converted to S—S at the junction of granular and horny layers. Such S—S rich zone may correspond to the marginal band [8,9] of horny cells. —SH groups and S—S covalent linkages were demonstrated neither in the keratohyaline granules nor in the trichohyaline granules by Barrnett [7] and Eisen [10]. By the use of DACM staining, these were also nonreactive in all the materials that we observed. Although trichohyaline granules of Henle and Huxley layers are gigantic, it is still possible that a strong fluorescence

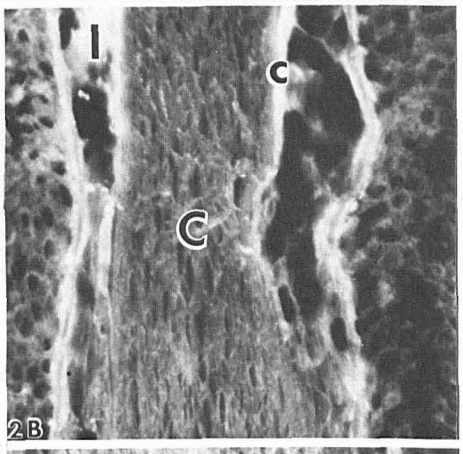


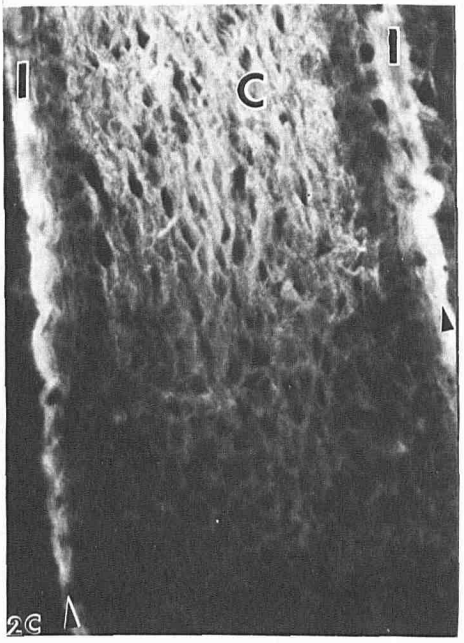












of Henle layer, for example, masked the identity of these granules.

In the hair a reciprocal staining pattern of —SH and S—S, as in the epidermis, was observed; namely, —SH is mainly localized in maturing but nonkeratinized living cells, whereas S—S linkages were seen in keratinized portion. In the bulb, inner root sheath and outer root sheath, which were rapidly maturing or relatively mature, always showed the presence of —SH groups; the hair cortex and hair medulla, which are still dividing or defectively keratinizing, contained less —SH groups. Barnett [7], Eisen, Montagna, and Chase [10] and Montagna et al [11] reported similar results on —SH staining of the bulb. Montagna et al [11] reported, however, that the distribution of S—S linkages in the outer and inner root sheaths of hair follicle is similar to that of SH groups. In contrast, our results suggest that outer root sheath, inner root sheath and hair cortex have little S—S covalent linkages in the bulb.

At the region of keratogenous zone, inner root sheath and hair cortex seem to convert —SH to S—S, but outer root sheath was still nonreactive for S—S. Ultrastructurally outer root sheath does not keratinize up to the isthmus where a few layers of weakly keratinized cells line the hair canal and sebaceous duct [12]. Corresponding to this change, S—S groups became reactive in the isthmus along the hair canal; apparently —SH was converted to S—S concomitant with keratinization. Persistence of —SH in the debris of inner root sheath, sebaceous duct, and in medullary portion of the cortex may suggest that some components of these structures are not completely keratinized. The distributions of S—S linkages in other layers correlated well with the keratinization of each layer as determined by electron microscopy [12] and support the general rule stated above that S—S linkages are formed concomittantly with cellular keratinization.

A previous report of Montagna et al [11] indicated that in the cortex —SH staining of the keratogenous zone was more intense than in the bulb and disappeared suddenly at the keratinized portion of the hair shaft. Odland [13] reported the same behavior of —SH groups. In the present study using

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FIG 1. *A*, In the epidermis bright fluorescence for —SH is limited to the cytoplasm of viable layers: horny layer (*h*) is nonfluorescent (× 100). *B*, Ismuth-infundibulum. —SH. Outer root sheath (*o*) as well as the cuticle of hair (*arrows*) are strongly fluorescent. Sebaceous glands are fluorescent along the cell periphery but keratinized sebaceous duct (*d*) is strongly fluorescent. Hair cortex (*c*) is moderately and irregularly fluorescent particularly in the central portion (reduced from × 40). *C*, Upper keratogenous zone-isthmus —SH. Outer root sheath (*o*) and the cuticle of cortex (*arrow*) are strongly positive. The cortex is uniformly fluorescent in upper keratogenous zone (*). Toward isthmus it fluoresces irregularly in less intensity except in the center. Debris of inner root sheath is strongly fluorescent (*arrowhead*). cf. Fig 2*A*, 2*B* (reduced from × 40). *D*, Bulb-keratogenous zone. —SH. Fluorescence increases in intensity from upper bulb to keratogenous zone (*k*). Inner root sheath (*i*) is moderately to strongly reactive. Hair papilla (*p*) is nonreactive. Outer root sheath (*o*) begins to react from upper bulb region (reduced from × 40).

FIG 2. *A*, Above keratogenous zone-isthmus. —SH. Detached inner root sheath cells (*I*) comprised of Henle, Huxley and the cuticle are moderately fluorescent. Cuticle of the cortex (*c*) is also moderately fluorescent, while the cortex (*C*) is only irregularly reactive. *O*: outer root sheath. cf. Fig 1*C* (reduced from × 200). *B*, Upper keratogenous zone. —SH. This is a lower portion of Fig 2*A*. Cortex (*C*) is more fluorescent than in Fig 2*A*. Cuticle of the cortex (*c*) and debris of the inner root sheath (*I*) are already strongly fluorescent. *O*: outer root sheath (reduced from × 200). *C*, Keratogenous zone. —SH. Cortex (*C*) and inner root sheath (*I*) begin to react at lower keratogenous zone. Fluorescence in the inner root sheath seems to begin in outer layers (Henle, *arrowheads*), while the inner portion (Huxley's) is less fluorescent (reduced from × 200).

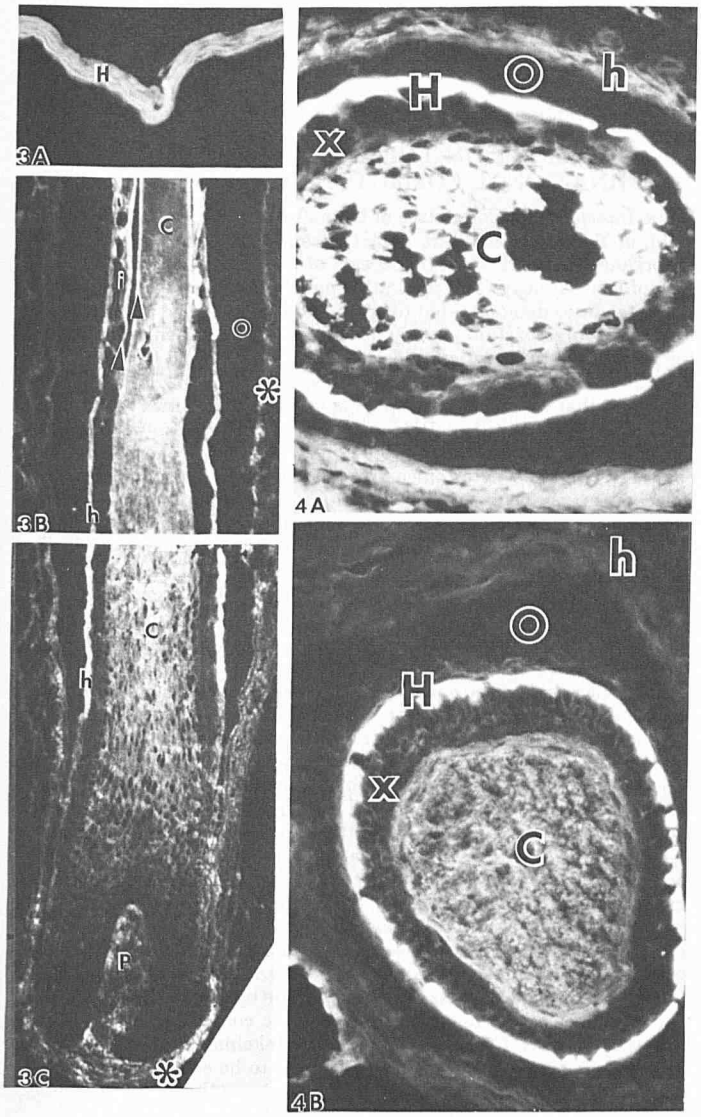

FIG 3. *A*, Epidermis. S—S. Strong fluorescence is only present in the horny layer (*H*) (reduced from × 100). *B*, Upper keratogenous zone-isthmus. S—S. Toward the isthmus (upper end of the picture) the cuticle of cortex and that of the inner root sheath could be separated as strongly fluorescent layers (*arrowheads*). Also, in the upper part of this picture above (*i*) not only Henle layer but also Huxley layer is fluorescence, whereas in the lower part only Henle layer (*h*) is reactive. Cortex (*C*) is strongly reactive. Outer root sheath (*O*), which is completely negative, is surrounded with moderately reactive hyaline sheath (*) (reduced from × 100). *C*, Bulb. S—S. Except hyaline sheath (*) and hair papilla (*P*), this region is entirely negative for S—S. At the keratogenous zone Henle layer (*h*) and cortex (*C*) become fluorescent. Nonreactive zone between *h* and *c* represents Huxley layer and cuticles (reduced from × 100).

FIG 4. *A*, Above the keratogenous zone. S—S. Henle layer (*H*) and Cortex (*C*) are very strongly fluorescent. Hyaline membrane (*h*) and Huxley layer (*X*) are moderately reactive. Outer root sheath (*O*), which is not keratinized at this level is entirely negative (reduced from × 200). *B*, Keratogenous zone. S—S. Cortex (*C*) begins to fluoresce, while Henle layer (*H*) is already very strongly fluorescent. Hyaline membrane (*h*) and Huxley layer (*X*) are moderately reactive. *O*: outer root sheath (reduced from × 200).

DACM, we found that these —SH stains were decreased rather gradually above the keratogenous zone. Also, at the region of isthmus or infundibulum the cortex still had a moderately strong fluorescence by —SH stain, particularly in the center where incompletely keratinized medulla may persist. It has been believed that the conversion of —SH to S—S is mediated by a sulfhydryl oxidase [14]. It is possible that this process progresses continuously in the cortex after an acute onset at a lower level of keratogenous zone. At the region of lower keratogenous zone S—S linkages were seen only in Henle's layer. Subsequently cortex of the hair, cuticle and Huxley's layer become positive above the keratogenous zone. Hashimoto and Shibazaki [12] summarized observations on hair keratinization at fine structural level and described that Henle's layer keratinizes earliest not only among 3 layers of the inner root sheath but in the entire hair follicle. Our histochemical demonstration of conversion of —SH to S—S agrees with this observation.

As mentioned above, there are several discrepancies between the results obtained by DACM method and previous histochemical methods. We believe that the findings obtained by DACM stain is more specific. Other methods for detection of sulfhydryl groups are based on reactions with oxidizing, reducing, alkylating or mercaptide-forming reagents. Most of them lack specificity for —SH groups because functional groups other than —SH are quite reactive toward these reagents.

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